PB82-154857

Morphological Effects of Prolonged Exposure to Ozone and Sulfuric Acid Aerosol on the Rat Lung

California Univ., Davis

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

5 Jan 81

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA (Please read Instructions on the reverse before completing)		XX-82-001
1. REPORT NO. EPA-600/J-81-519	2. JOURNAL ARTICLE	3. PB82-154857
4. TITLE AND SUBTITLE Morphological Effects of Prolonged Exposure to Ozone Acid Aerosol on the Rat Lung		5. REPORT DATE January 1982 6. PERFORMING ORGANIZATION CODE 2025104
7. AUTHOR(S) P.F. Moore and L.W. Schwartz		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Veterinary Pathology and California Primate Research Center University of California at Davis Davis, California 95616		10. PROGRAM ELEMENT NO. C9RA1B 11. CONTRACT/GRANT NO. Contract No: 68-02-2496
12. SPONSORING AGENCY NAME AND ADDRESS Office of Research and Development (RTP,NC) Health Effects Research Laboratory US Environmental Protection Agency Research Triangle Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA-600/11
15. SUPPLEMENTARY NOTES Published In: Experimental and Molecular Pathology 35: 108-123, 1981		

16. ABSTRACT

The purpose of this study was to determine the pulmonary effects of a combination of ozone (0.5 ppm) and sulfuric acid aerosol (1 mg/m^3) and to assess the possibility of interactive effects. Groups of Sprague-Dawley rats were continously exposed to the pollutants, either individually of combined, for 3, 50, 90, or 180 days. After 180 days of exposure, additional groups breathed clean air for a further 62 days. Morphological evaluation included light microscopy, autoradiography, scanning electron microscopy (SEM), and transmission electron microscopy. Quantification of pulmonary centriacinar inflammatory cell response was performed by SEM. The results clearly demonstrated that exposure to 0.5 ppm ozone for 180 days resulted in a persistent inflammatory response in the pulmonary centriacinar region together with a structural modification of the terminal bronchiole - proximal alveolar duct junction. Sulfuric acid aerosol did not induce pulmonary morphological changes, nor did it potentiate lesions produced by simultaneous ozone exposure. After termination of the 62-day postexposure period, ozone and ozone plus sulfuric acid postexposure rats demonstrated a marked diminution in the intensity of the pulmonary centriacinar inflammatory response and a partial restoration of normal centriacinar structure.

17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS (This Report) UNCLASSIFIED	21. NO. OF PAGES 18
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE

EPA-600/J-81-519
Journal Article

EXPERIMENTAL AND MOLECULAR PATHOLOGY 35, 108–123 (1981)

Morphological Effects of Prolonged Exposure to Ozone and Sulfuric Acid Aerosol on the Rat Lung

P. F. MOORE AND L. W. SCHWARTZ

Department of Veterinary Pathology and California Primate Research Center, University of California, Davis, California 95616

Received September 30, 1980, and in revised form February 5, 1981

The purpose of this study was to determine the pulmonary effects of a combination of ozone (0.5 ppm) and sulfuric acid aerosol (1 mg/m^3) and to assess the possibility of interactive effects. Groups of Sprague–Dawley rats were continuously exposed to the pollutants, either individually or combined, for 3, 50, 90, or 180 days. After 180 days of exposure, additional groups breathed clean air for a further 62 days. Morphological evaluation included light microscopy, autoradiography, scanning electron microscopy (SEM), and transmission electron microscopy. Quantification of pulmonary centriacinar inflammatory cell response was performed by SEM. The results clearly demonstrated that exposure to 0.5 ppm ozone for 180 days resulted in a persistent inflammatory response in the pulmonary centriacinar region together with a structural modification of the terminal bronchiole–proximal alveolar duct junction. Sulfuric acid aerosol did not induce pulmonary morphological changes, nor did it potentiate lesions produced by simultaneous ozone exposure. After termination of the 62-day postexposure period, ozone and ozone plus sulfuric acid postexposure rats demonstrated a marked diminution in the intensity of the pulmonary centriacinar inflammatory response and a partial restoration of normal centriacinar structure.

INTRODUCTION

Much of the past work on air pollutants has been limited to single pollutant exposure regimens. Current investigative trends are toward determining health effects of pollutant combinations in an attempt to identify potential synergistic effects that might be operative in complicated natural pollution situations.

Concern over health effects of sulfur oxides and particulates has been reinforced by the present energy situation, which is accompanied by the likelihood of increased utilization of fossil fuels containing high concentrations of sulfur to meet energy needs. The lower oxides of sulfur (SO_2, SO_3) are convertible in the atmosphere and in the automobile catalytic converter to the higher oxides, sulfuric acid and sulfate (Amdur, 1970; Pierson *et al.*, 1974). Previous investigations have indicated that these higher oxides of sulfur have more deleterious effects on the respiratory tract than the lower oxides at equivalent concentrations (Amdur, 1970, 1974). Ozone continues to be of major importance as the principal oxidant of photochemical smog (Hagen-Smit, 1952). Exposure to ambient concentrations induces both acute and chronic morphological damage, particularly in the centriacinar region of the lung. A comprehensive review of the health effects of ozone has been published by Dungworth and co-workers (Dungworth *et al.*, 1975).

Our major objectives in this study were twofold: first, to explore the prolonged effects on rats of a combination of ozone and sulfuric acid aerosol at realistic levels, and second, to determine if sulfuric acid was capable of potentiating ozone-induced lung damage. To this end, systematic quantitative methods were used whenever possible. An ozone concentration of 0.5 ppm was chosen to represent a high ambient level. This ozone concentration is reached and exceeded on occasion in certain portions of the California South Coast Air Basin (Air Re-

sources Board, 1974). A sulfuric acid mass concentration of 1 mg/m^3 was chosen since it represents the current National Institute of Occupational Safety and Health occupational maximum standard for an 8-hr day, even though this concentration is up to 50-fold higher than reported ambient levels (Juhos *et al.*, 1978).

MATERIALS AND METHODS

Animals and Animal Handling

Male Sprague–Dawley rats, 70 ± 3 days old, were obtained from a colony free of murine respiratory mycoplasmosis (Hilltop, Scottsdale, Pa.). They were housed two per cage under microbiologic filters for 5 days prior to exposure and were provided water and Purina lab chow *ad libitum*.

For exposure, rats were housed in pairs in open-mesh stainless-steel wire cages within 4.2-m^3 chambers fabricated from stainless steel and glass as described by Hinners and associates (Hinners *et al.*, 1968). Each chamber contained a single layer of 25 cages. The rats were individually identified by numbered ear tags and weighed prior to exposure. They were reweighed immediately prior to sacrifice. The animal volume was about 0.6% of the chamber volume. Food and water were again provided *ad libitum*. Cleaning was performed once daily. Air supplied to each chamber was chemical, bacteriological, and radiological filtered (CBR) (Model CV85240 C-B-R filter, Mine Safety Appliance Co., Evans City, Pa.). Air flow was 2.1 m^3/min. Temperature was maintained at 23 ± 1°C and relative humidity at about 50%.

Exposure Procedure

Rats were randomly divided into four groups; each group contained 100 rats, which occupied two chambers. The respective groups were continuously exposed to one of the following atmospheres: CBR-filtered air; CBR-filtered air containing 0.5 ppm ozone; CBR-filtered air containing 1 mg/m^3 sulfuric acid; or CBR-filtered air containing 0.5 ppm ozone and 1 mg/m^3 sulfuric acid. Six rats from each group were sampled for morphologic studies at 3, 50, 90, and 180 days after exposure commenced. After the 180-day exposure period an additional six rats from each group were removed to an isolation room and were housed in pairs under microbiologic filters for a 62-day postexposure evaluation period. The remaining rats were used by other investigators for biochemical and physiological studies.

Sulfuric acid aerosol was generated from liquid sulfur trioxide. The details of the generation and monitoring system for sulfuric acid have been described in detail elsewhere (Chang and Tarkington, 1977; Chang *et al.*, 1978). Ozone was produced from medical grade oxygen by silent arc discharge ozonizers. The ozone generation and monitoring procedures have been described in detail previously (Schwartz *et al.*, 1976). The exposure data for this study are summarized in Table I.

Tissue Preparation for Morphologic Evaluation

One hour prior to death all rats were labeled with 1 μCi/g body wt of tritiated thymidine ([^{3}H]dT; specific activity 6.7 Ci/m*M*; New England Nuclear, Boston, Mass.) by intraperitoneal (ip) injection. Rats were anesthetized with sodium pentobarbital (Diabutal; 60 mg ip) and exsanguinated by cardiac puncture. The lower trachea and lungs were removed by thoracotomy and inflated via the trachea with modified Karnovsky's fixative at 30 cm of fixative pressure. The larynx and upper

TABLE I
Summary of Exposure Data[a]

Group	Chamber	Days	H_2SO_4 (mg/m^3)[b]	CMD (μm)[c]	ρg[d]	O_3 (ppm)
H_2SO_4	4.2-1	3	1.03 ± 0.61			ND[e]
		50	1.20 ± 0.33			
		90	1.21 ± 0.27			
		180	1.22 ± 0.25	0.032 ± 0.004	1.8 ± 0.10	
	4.2-2	3	0.66 ± 0.16			
		50	1.15 ± 0.35			
		90	1.23 ± 0.31			
		180	1.24 ± 0.26	0.044 ± 0.007	2.1 ± 0.32	
$H_2SO_4 + O_3$	4.2-3	3	0.80 ± 0.18			0.53 ± 0.04
		50	1.12 ± 0.24			0.51 ± 0.04
		90	1.09 ± 0.23			0.51 ± 0.04
		180	1.13 ± 0.23	0.035 ± 0.001	1.8 ± 0.21	0.51 ± 0.04
	4.2-4	3	0.75 ± 0.05			
		50	1.14 ± 0.31			0.50 ± 0.04
		90	1.16 ± 0.25			0.52 ± 0.04
		180	1.19 ± 0.24	0.036 ± 0.007	1.8 ± 0.17	0.50 ± 0.04
O_3	4.2-7	3	ND[e]			0.50 ± 0.03
		50				0.50 ± 0.03
		90				0.49 ± 0.03
		180				0.49 ± 0.03
	4.2-8	3				
		50				0.49 ± 0.03
		90				0.49 ± 0.03
		180				0.48 ± 0.03

[a] All data are expressed as mean ± standard deviation.
[b] Mass concentration determined from daily filter samples.
[c] Count median diameter, which is the median of a distribution of particle diameters, was determined from cumulated data over the entire exposure period.
[d] Geometric standard deviation.
[e] Not determined.

trachea were fixed by immersion in modified Karnovsky's fixative. About 20 hr after the commencement of fixation the trachea was ligated just above the carina, and the portion of the trachea above the ligature as well as the entire esophagus, thymus, and heart were removed, leaving the lungs and a small tracheobronchial stump. The fixed lung volume was then determined by the fixative displacement method of Scherle (1970). The 95% confidence limits were computed using Student's *t* test.

The entire left lung lobe was sectioned in a vertical plane along the main bronchus with an alcohol-cleaned stainless-steel razor blade. Adjacent areas were selected for scanning electron microscopy (SEM) and epoxy resin embedding. The right caudal lobe was sectioned in a similar plane, and one-half was selected for routine light microscopic (LM) examination of paraffin-embedded tissue.

Scanning Electron Microscopy

Lung samples, no larger than 1 cm^2, which contained a longitudinal section of a conducting airway and/or one or more terminal bronchioles, were selected for

SEM. The methods of tissue preparation were similar to those of Schwartz *et al.* (1976).

All centriacinar regions, which consist of a segment of terminal bronchiole and proximal alveoli, were identified, photographed, and evaluated. Beginning at the most proximal alveolus and moving distally, the number of alveoli visible within the first alveolarized airway was counted and the number of inflammatory cells within this same region was recorded. Previous LM had indicated that these cells were predominantly alveolar macrophages. When clumps or aggregates of inflammatory cells were observed, only cells visible from the surface as individual units were counted. Data from each rat were pooled to yield a mean figure for inflammatory cells per alveolus for a particular exposure group. A minimum of five centriacinar regions from each rat contributed to this mean. The 95% confidence limits were computed using Student's *t* test.

Autoradiography

Lung tissue blocks, selected from the left lobe, were dehydrated in acetone and embedded in Epon–Araldite. One-micrometer sections were cut with glass knives on a JB4 microtome (Sorvall, Newtown, Conn.). The sections were dipped in Ilford L4 emulsion and exposed for 30 days at 4°C in light-tight boxes. The autoradiograms were developed, fixed, and then stained with 1% toluidine blue in 1% sodium borate.

Terminal bronchiolar cells were counted by LM using a 100× oil immersion objective and a total magnification of 1000×. Only bronchioles with a direct communication between conducting airway and alveolar duct were counted; this ensured that a similar level of airway was always evaluated. A minimum of 3000 bronchiolar cells were counted from each exposure group. Cells were counted only if they contained a section of nucleus and a luminal surface.

Terminal bronchiolar epithelial cells were classified into three major categories by LM of 1.0-μm sections, i.e., ciliated, nonciliated secretory (Clara), and nonciliated nonsecretory, according to criteria defined by Lum *et al.* (1978). A cell was considered labeled only if five or more silver grains overlay its nucleus. The labeling index (LI) was defined as the number of labeled cells per 1000 terminal bronchiolar cells. The 95% confidence limits were computed using Student's *t* test.

Transmission Electron Microscopy

Selected exposed and control tissues removed from the left lung lobe of 180-day and recovery rats were embedded in Epon–Araldite as described under autoradiography. Centriacinar regions were selected for thin sectioning after viewing 1-μm toluidine blue stained sections. Thin sections were cut on an MT2B ultramicrotome (Sorvall), stained with uranyl acetate and lead citrate, and viewed on a Zeiss EM 10 transmission electron microscope.

RESULTS

Lung Volume and Body Weight

Consistent exposure-related trends were not observed with respect to body weight. Age-related increases in lung volume were observed in control rats during the exposure period, especially in the interval between 3 and 50 days of exposure. The total lung volume was significantly larger than control in rats exposed to

ozone and ozone plus sulfuric acid for 180 days (Table II). The postexposure groups' lung volumes were not different from controls with the exception of the ozone-exposed rats, whose lung volumes were lower than control.

Morphology

The pulmonary morphology of the control rats was similar to that in previous descriptions (Schwartz *et al.*, 1976; Boorman *et al.*, 1980), with one exception; clusters of bronchiolar epithelial cells were occasionally found distal to the first alveolus in the centriacinar region (Figs. 1A, B, C). This change was most easily appreciated by SEM and did not alter appreciably in degree throughout the exposure and recovery periods. Because the principal site of ozone damage is at the junction of the conducting airways and exchange tissue, the centriacinar region, most attention was focused at this site.

Throughout this study morphological differences were never observed between control and sulfuric acid-exposed rats. Similarly, changes observed in the ozone plus sulfuric acid-exposed rats were indistinguishable from those of the ozone-only group and will therefore not be described separately from ozone-induced lesions.

The acute ozone-induced lesions in the centriacinar region after 3 days of exposure were consistent with previous published accounts (Schwartz *et al.*, 1976; Stephens *et al.*, 1974). Rats exposed to ozone for 50, 90, or 180 days had qualitatively similar terminal bronchiolar surface alterations consisting of flattening of nonciliated secretory cell apical cytoplasmic protrusions, variation in the number and length of cilia, and blebbing of nonciliated cells and cilia (Fig. 2A). Small clusters of nonciliated cells were observed in the terminal bronchiolar epithelium of most rats after 90 days ozone exposure (Fig. 2B), but were no longer evident after 180 days exposure. Instead, larger nodular aggregates of nonciliated cells, which bulged into the terminal bronchiolar lumen, were observed in only 2 of 12 ozone-exposed rats after 180 days (Fig. 2C).

A distinct structural modification was present in the airway immediately distal to the terminal bronchiole in all ozone-exposed rats after 50, 90, or 180 days. The severity of the change was greatest after 180 days ozone exposure. By SEM, control rats had terminal bronchioles in which the bronchiolar epithelium ended fairly abruptly, giving rise to an alveolar duct with thin interalveolar septa lined only by alveolar type 1 and type 2 epithelium, except for infrequent small clusters of bronchiolar epithelium described above. Instead, ozone-exposed rats had a

TABLE II
Total Lung Volume (cm^3) (mean ± SD)

Days exposed	Control	H_2SO_4	O_3	$H_2SO_4 + O_3$
3	12.5 ± 0.8	12.6 ± 0.4	13.1 ± 1.2	13.4 ± 1.1
50	16.5 ± 1.3	16.0 ± 1.2	17.8 ± 1.2	16.6 ± 0.9
90	17.6 ± 1.0	18.0 ± 2.1	18.6 ± 2.1	18.3 ± 0.9
180	17.4 ± 2.8	17.0 ± 1.9	21.4 ± 1.8**	20.4 ± 2.7*
62 Days postexposure	18.7 ± 1.4	18.7 ± 0.9	17.2 ± 0.9*	18.5 ± 1.8

* Indicates a significant difference between the exposure group and the control group (* $P < 0.05$; ** $P < 0.01$).

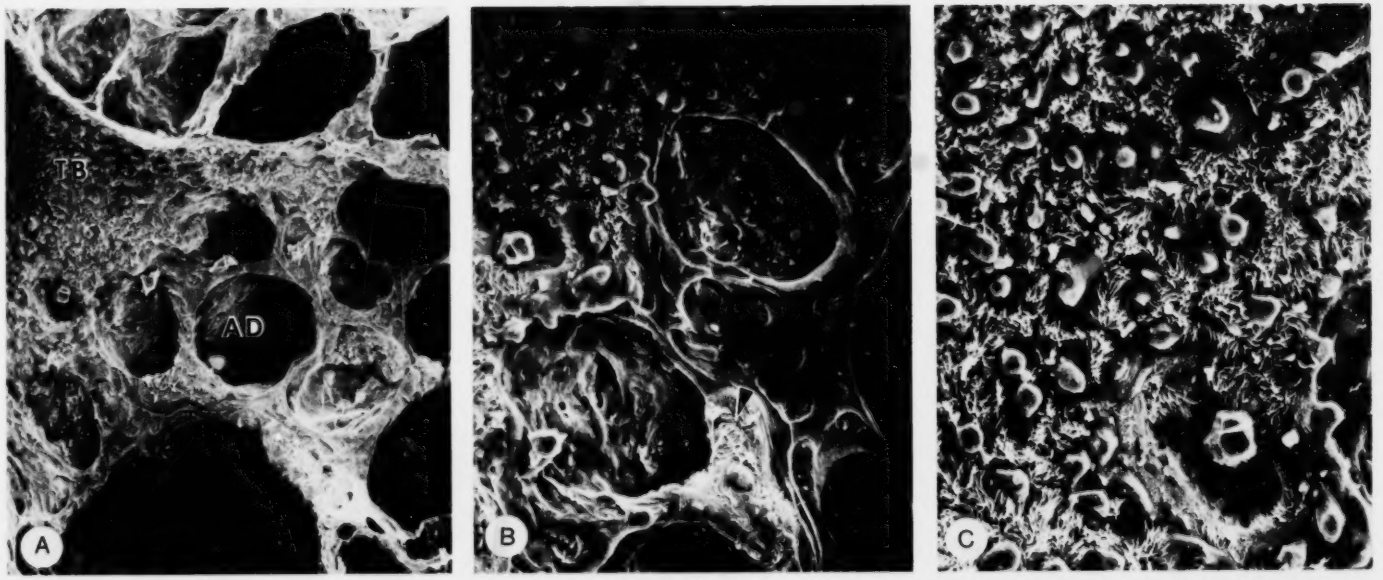

FIG. 1. One hundred and eight-day control rat pulmonary centriacinar region. (A) Note the abrupt termination of the terminal bronchiole (TB). Only occasional isolated patches of bronchiolar epithelium are visible in the proximal alveolar duct (AD). SEM; 400×. (B) Higher magnification of the terminal bronchiole – alveolar duct junction of (A) illustrating the alveolar-type epithelium of the alveolar duct and an isolated patch of bronchiolar epithelium in the proximal alveolar duct (arrowhead). SEM; 700×. (C) Higher magnification of (A) illustrating normal terminal bronchiolar epithelium. Note the prominent smooth apical cytoplasmic projections of nonciliated cells and the regularly interspersed ciliated cells. SEM; 1000×.

FIG. 2. Terminal bronchiolar surface changes in ozone plus sulfuric acid-exposed rats. (A) 180 days exposed. Note the flattening and blebbing of the apical cytoplasmic protrusions of nonciliated cells. Ciliated cells exhibit marked variations in the number and length of cilia. SEM; 1000×. (B) 90 days exposed. Note the small clusters of flattened and blebbed nonciliated cells. SEM; 2000×. (C) 180 days exposed. Note the larger nodular aggregates of nonciliated cells. This change was observed in only two rats. SEM; 1000×.

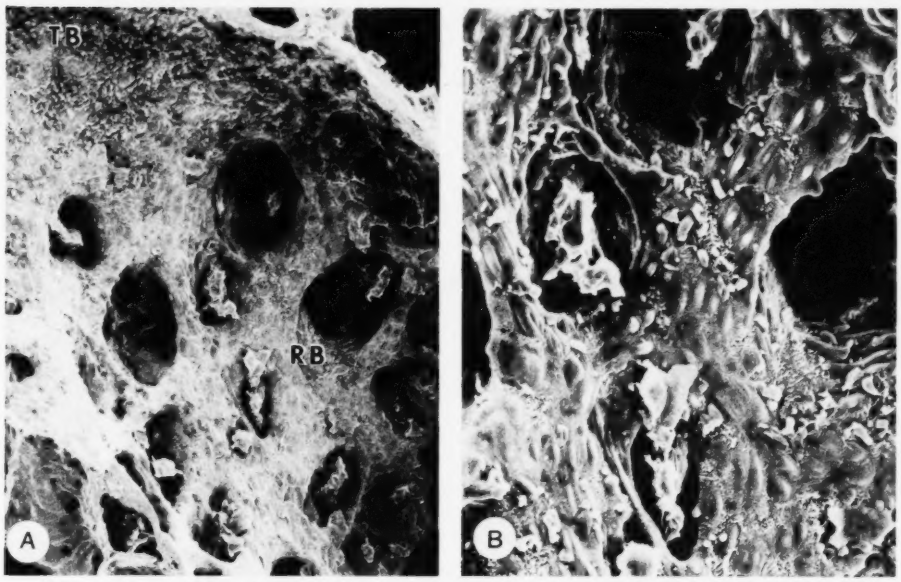

FIG. 3. Structural alteration of the centriacinar region of ozone plus sulfuric acid-exposed rats after 180 days exposure. (A) Note the segment of respiratory bronchiole (RB) distal to the terminal bronchiole (TB). The alveolar epithelium normally lining the septa between the proximal alveoli has been replaced by bronchiolar epithelium. Clusters of macrophages occupy most alveoli. SEM; 400×. (B) Higher magnification of the respiratory bronchiole segment of (A) illustrating flattening of bronchiolar nonciliated cell apical processes and the short disoriented cilia of ciliated cells. Bronchiolar epithelium extends into alveoli, which also contain clusters of alveolar macrophages. SEM; 800×.

segment of airway in which the proximal alveoli were separated by thick interalveolar septa (Fig. 3A); the septa were lined completely by bronchiolar epithelium that extended into adjacent alveoli. These alveoli were not only lined by bronchiolar epithelium, but also by alveolar epithelium that was not interspersed within bronchiolar epithelium. Nonciliated cells in this segment of airway were uniformly flattened. Ciliated cells were sparse and possessed short disoriented cilia. Clusters of macrophages often occupied the proximal alveoli (Fig. 3B). Nonciliated cells lining the proximal interalveolar septa and proximal alveoli often contained densely osmiophilic apical bodies typical of the secretory granules in nonciliated secretory bronchiolar cells (Fig. 4A). Other cells in the same sites exhibited numerous basal bodies in the apical cytoplasm, yet had few or no cilia (Fig. 4A). Cuboidal cells possessing neither basal bodies nor secretory granules were often seen. These cells had abundant granular endoplasmic reticulum, numerous mitochondria, and a microvillous surface (Fig. 4B).

The majority of rats from the ozone exposure groups had very mild interstitial thickening in the centriacinar region. Van Gieson's stain for collagen applied to paraffin sections indicated the interstitial thickening was due to collagen deposition. The degree of this change differed markedly between different centriacinar regions in the same animal. Airways intensely infiltrated by macrophages tended to have a higher frequency of occurrence of accompanying fibrotic changes.

The ozone postexposure rats had substantially different pulmonary morphology

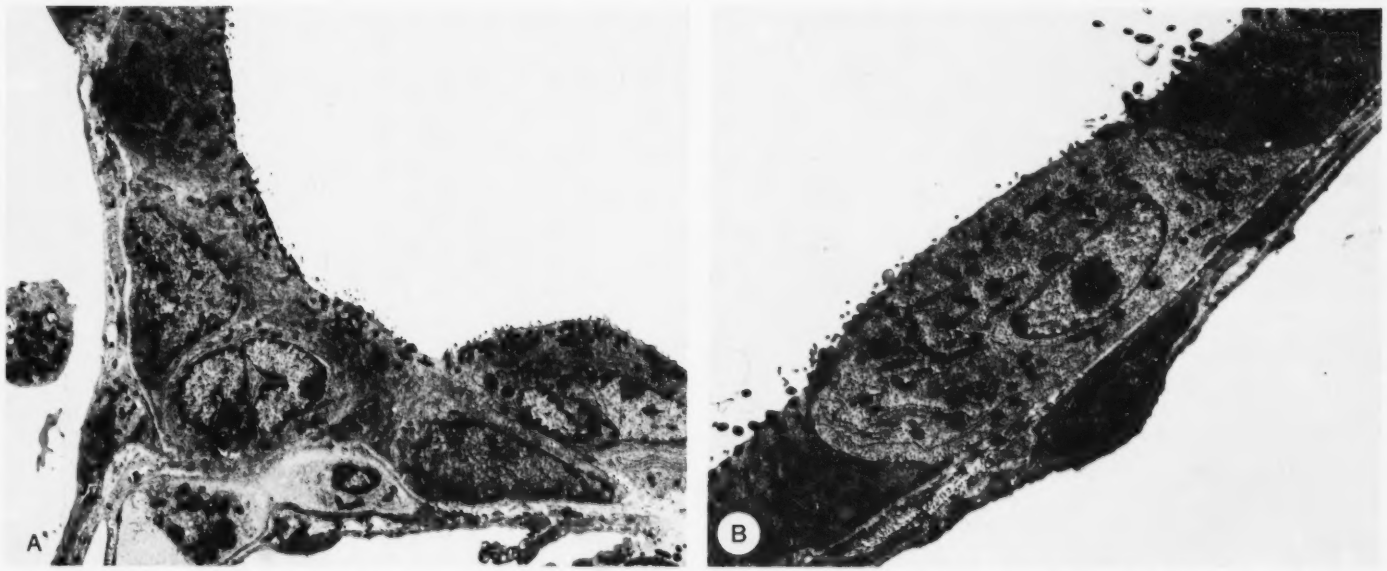

FIG. 4. Ultrastructural features of epithelial cells lining proximal alveoli of the segments of respiratory bronchiole. 180-day ozone plus sulfuric acid-exposed rat. (A) Bronchiolar-type epithelium. Note secretory granules (solid arrow), disoriented basal bodies, and lack of cilia (open arrow). TEM; 4300×. (B) Cuboidal cell lacking basal bodies and secretory granules. TEM; 6500×.

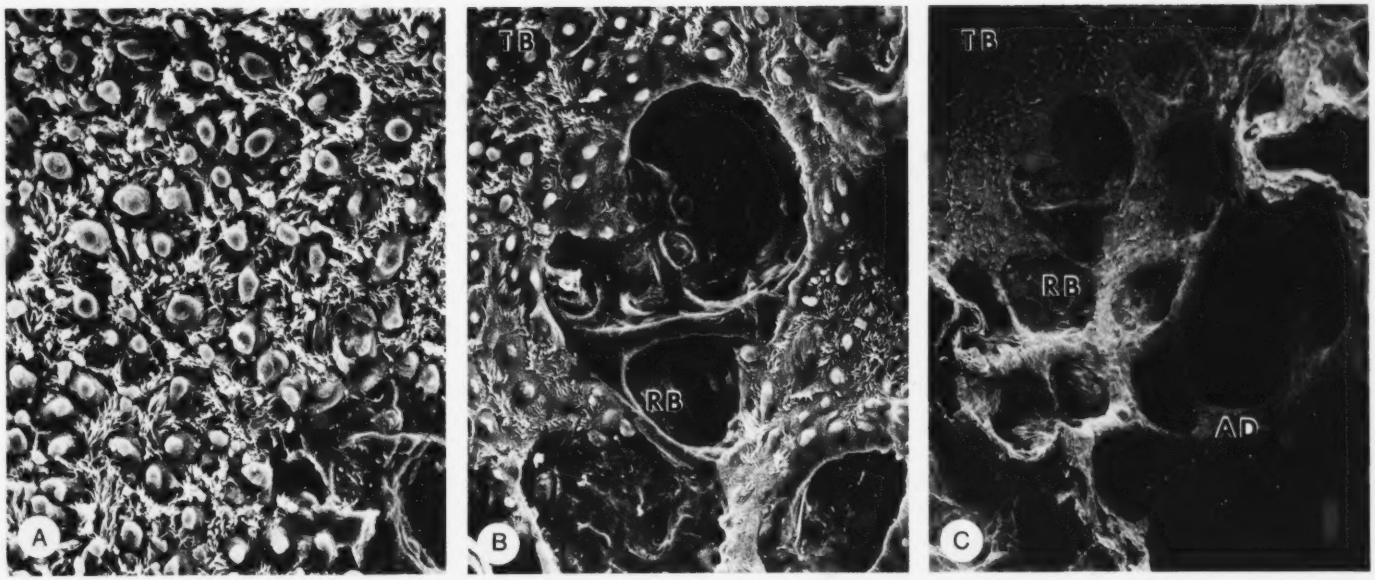

FIG. 5. Centriacinar morphology of the ozone plus sulfuric acid postexposure group. (A) Terminal bronchiolar surface morphology comparable to control. SEM; 1000×. (B) Terminal bronchiole (TB)–respiratory bronchiole (RB) junction illustrating some residual flattening of nonciliated cells. The microvilli on these same cells are difficult to discern at this magnification. SEM; 700×. (C) Lower magnification of (B) illustrating the diminished length of the segment of respiratory bronchiole (RB), sandwiched between the terminal bronchiole (TB) and the alveolar duct (AD). Compare the length of the RB segment to the same in Fig. 3A, which is printed at the same magnification. SEM; 400×.

from their 180-day exposed counterparts. The most striking difference was in the intensity of the infiltration of mononuclear cells into the centriacinar region. Ozone postexposure rats had only slightly increased macrophage infiltration when compared with controls. By SEM the terminal bronchiolar surface was comparable to control terminal bronchiole (Fig. 5A) with a few exceptions in which there were some residual flattening of nonciliated cell apical cytoplasmic projections and increased numbers of cells covered by microvilli (Fig. 5B).

The structural modification of the airway segment immediately distal to the terminal bronchiole was comparable qualitatively to that observed after 180 days ozone exposure; however, the length of this airway segment was much reduced in ozone postexposure rats (Fig. 5C). LM of Van Gieson stained paraffin sections revealed that the amount and extent of the centriacinar collagen deposits was less than that observed after 180 days ozone exposure.

SEM Quantification of the Centriacinar Inflammatory Response

The results of this analysis are summarized in Table III. The cell counts of ozone and ozone plus sulfuric acid-exposed rats were not significantly different from each other throughout the exposure or postexposure period. Likewise, cell counts of control and sulfuric acid-exposed rats did not differ. Rats exposed to ozone had significantly elevated cell counts throughout the exposure period. The peak of cellularity occurred after 50 days exposure (mean of the two ozone groups: 11 inflammatory cells per alveolus). Cellularity was still quite elevated after 180 days exposure (mean: 7.6 inflammatory cells per alveolus). Cessation of exposure brought about a dramatic decrease in inflammatory cell counts in the ozone-exposed group (mean: 1.2 inflammatory cells per alveolus). The control group, however, had a slightly lower cell count (0.7 inflammatory cells per alveolus). This quantitative approach of counting inflammatory cells per alveolus indicated a significant difference between the control and ozone postexposure rats, but using only subjective evaluation of scanning electron micrographs we generally failed to distinguish control rats from exposed rats in postexposure groups.

Terminal Bronchiolar Epithelial Cell Turnover

The results of the autoradiography, expressed as a labeling index (LI), are summarized in Fig. 6. The LI of control terminal bronchiolar epithelium was 1.68,

TABLE III
Intensity of the Inflammatory Cell Response in the Centriacinar Region
(Mean of Inflammatory Cells per Alveolus ± SD)

Days exposed	Control	H_2SO_4	O_3	$H_2SO_4 + O_3$
3	0.5 ± 0.1	0.5 ± 0.1	4.0 ± 0.4**	4.0 ± 0.6**
50	1.3 ± 0.6	1.3 ± 0.4	11.1 ± 1.0**	11.0 ± 1.1**
90	1.1 ± 0.2	1.0 ± 0.1	8.3 ± 1.1**	8.2 ± 0.9**
180	1.0 ± 0.1	1.1 ± 0.1	7.8 ± 1.8**	7.4 ± 2.1*
62 days postexposure	0.7 ± 0.1	0.8 ± 0.1	1.1 ± 0.1**	1.2 ± 0.4*

* Indicates a significant difference between the exposure group and the control group (* $P < 0.05$, ** $P < 0.001$).

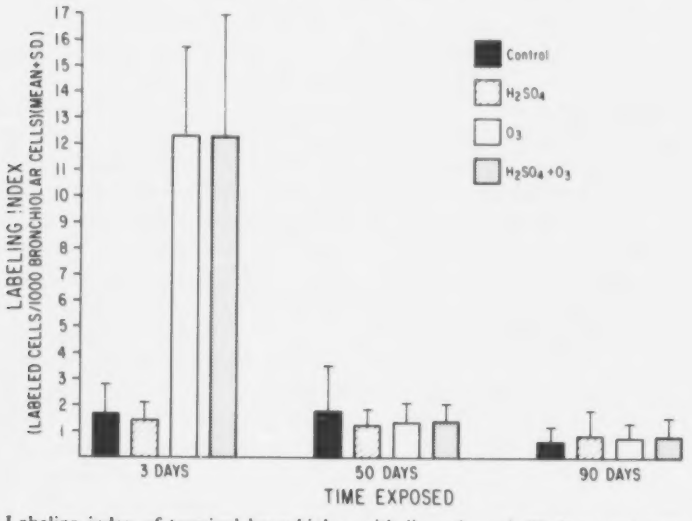

FIG. 6. Labeling index of terminal bronchiolar epithelium through 90 days exposure. Labeling indices of ozone and ozone plus sulfuric acid-exposed rats were significantly different from control only after 3 days exposure ($P < 0.01$).

1.78, and 0.63 after 3, 50, and 90 days exposure, respectively. The nonciliated secretory cell was the predominant labeled cell type (80% of labeled cells). The sulfuric acid-exposed groups' labeling indices were never significantly different from control. Labeling indices of ozone and ozone plus sulfuric acid-exposed rats were not significantly different from each other after 3, 50, and 90 days exposure. There was about a sevenfold increase in LI after 3 days exposure in the ozone-exposed groups (mean LI of the two ozone groups: 12.3). After 50 and 90 days exposure, however, the labeling indices of both ozone-exposed groups were not significantly different from the respective control values. The predominant labeled cell in the ozone-exposed rats' terminal bronchiole was the nonciliated secretory cell (mean of the two ozone groups: 66, 73, and 60% of labeled cells after 3, 50, and 90 days exposure, respectively). Nonciliated nonsecretory cells were only labeled substantially in both ozone-exposed groups (25, 27, and 40% of labeled cells after 3, 50, and 90 days exposure, respectively). Labeled ciliated cells were seen infrequently. Autoradiographic studies were discontinued after 90 days exposure because there were no longer differences between control and ozone-exposed rats.

DISCUSSION

This study clearly demonstrates that exposure to ozone at a concentration of 0.5 ppm for 180 days results in a persistent inflammatory response in the centriacinar region of the lung together with a structural modification of the junction between the terminal bronchiole and the alveolar duct. Significant exposure-related changes were not observed in sulfuric acid-exposed animals at any time during the exposure period. Pulmonary changes caused by exposure to the combination of ozone plus sulfuric acid were attributable solely to ozone exposure; potentiating effects of sulfuric acid on ozone-induced pulmonary changes were never observed throughout the 180-day exposure period. Ozone and ozone plus sulfuric acid-

exposed rats allowed to breathe clean air for a further 62 days had a marked diminution in the intensity of the centriacinar inflammatory response and a partial restoration of normal centriacinar region morphology.

The dominant feature of 180-day ozone-exposed rat lungs was the structural modification of the centriacinar region. In essence it consisted of the formation of a segment of respiratory bronchiole between recognizable terminal bronchiole and alveolar duct. This change has been recorded previously following prolonged exposure to ozone (Boorman *et al.*, 1980) and nitrogen dioxide (Kleinerman and Cowdrey, 1966). It seems likely that respiratory bronchiole formation in this instance is a form of "bronchiolization of alveoli," which has been observed to follow a variety of insults to the lung including respiratory infection, circulatory disorders, chemical irritants, and carcinogens (Nettesheim and Szakal, 1972). Several mechanisms may explain the existence of bronchiolar epithelium in situations where alveolar epithelium normally resides; they include metaplasia of alveolar type 2 epithelium (Adamson and Bowden, 1977); bronchiolar epithelial downgrowth on to alveolar septa of alveolar ducts or into peribronchiolar alveoli through porosities in the bronchiolar wall (Nettesheim and Szakal, 1972); and "alveolarization" of the terminal bronchiole, which has been described as a normal phenomenon in early postnatal development and in aging of the lung in a variety of species (Boyden and Tompsett, 1961, 1965; Burri, 1974; Burri *et al.*, 1974). A choice to include one or more of these mechanisms cannot be made with assurrance in the present study due to the prolonged intervals between animal samplings and the absence of bronchial generation count data. It is tempting, however, to speculate that bronchiolar epithelium in the proximal alveolar duct arose from disordered differentiation of persistently irritiated alveolar type 2 cells. Alveolar type 2 cell hyperplasia, as observed in this and other studies (Stephens *et al.*, 1974; Schwartz *et al.*, 1976) after about 3 days ozone exposure, resulted in a progeny intermediate in morphological appearance between alveolar type 1 and type 2 cells. These cells lined the alveoli and interalveolar septa of the proximal alveolar duct. Persistent ozone-induced irritation of the "intermediate" cell population might be expected to encourage differentiation to bronchiolar rather than to alveolar type 1 epithelium. An analogous situation has already been documented during bleomycin-induced alveolar epithelial injury (Adamson and Bowden, 1977). The functional implications of respiratory bronchiole formation are largely unknown; however, the fact that the lesion appears to be at least potentially reversible after cessation of ozone exposure is a testimony to the remarkable ability of the pulmonary epithelium to repair itself even after prolonged insult.

Persistent inflammation in the centriacinar region is potentially hazardous to the lung because of the ability of alveolar macrophages to secrete neutral proteases capable of utilizing pulmonary connective tissues as substrates (Werb and Gordon, 1975a, b; White *et al.*, 1977). Potent serum-derived and lung specific protease inhibitors exist in lung lavage fluid (White *et al.*, 1977) and probably mitigate the destructive potential of neutral proteases. Morphological evidence of chronic obstructive pulmonary disease, the expected outcome of unencumbered neutral protease activity in lung, was not evident in the lungs of ozone-exposed rats in this study. This suggests that the balance between neutral protease secretion rates and lung protease inhibitor levels may be a critical factor in determining the outcome of persistent inflammation in the pulmonary centriacinus and deserves further study.

Terminal bronchiolar epithelial changes after 180 days ozone exposure were largely restricted to subtle cell surface alterations best appreciated by SEM. This finding contrasts with the findings in studies using mice in which hyperplastic nodules of nonciliated cells were an early developing lesion in the terminal bronchiole after ozone exposure and persisted after exposure ceased (Bruch and Shlipköter, 1973; Penha and Werthamer, 1974; Zitnik *et al.*, 1978). An occasional rat, as demonstrated in this study, can develop hyperplastic nodules of nonciliated cells in the terminal bronchiole similar to those seen with more frequency and much earlier after ozone exposure in the mouse. Duration of insult may be an important factor contributing to this particular difference, although it is surely not the only one.

The absence of exposure-related lesions due to sulfuric acid and the absence of a potentiating effect of sulfuric acid on ozone-induced pulmonary lesions are consistent with the findings of previous morphological studies (Juhos *et al.*, 1978; Schwartz *et al.*, 1977; Cavender *et al.*, 1977, 1978). The present study, however, is the first to apply quantitative morphological techniques to the assessment of acute and chronic effects of a combination of ozone and sulfuric acid. The acid-neutralizing potential of ammonia generated within animal exposure chambers, presumably from bacterial action on urine and feces (Barrow and Dodd, 1979), must be considered in explaining the absence of sulfuric acid effects. In the present study, chamber airflow, cleaning procedures, and animal volume were designed to minimize the accumulation of ammonia within exposure chambers. The peak ammonia concentration, which occurred 4 hr after cleaning the chambers, was always less than 86 $\mu g/m^3$ (mean: 53 $\mu g/m^3$). Hence exposure chamber ammonia concentrations taken alone were insufficient to neutralize all the sulfuric acid, since ammonia can convert 5.8 times its own weight of sulfuric acid to ammonium bisulfate (Larson *et al.*, 1977). Ammonia is also excreted in the exhaled breath of mammals (Larson *et al.*, 1977; Loscutoff *et al.*, 1978; Barrow and Steinhagen, 1980). The concentration of ammonia in the exhaled air of rats has been quantified. The range for nose breathing rats was 7 to 250 $\mu g/m^3$ (mean: 55 $\mu g/m^3$) and for tracheal cannulated rats it was 24 to 526 $\mu g/m^3$ (mean: 202 $\mu g/m^3$) (Barrow and Steinhagen, 1980). These data taken together suggest that the potential for complete neutralization of the exposure concentration of 1 mg/m^3 sulfuric acid probably existed and could explain the lack of sulfuric acid-induced pulmonary lesions. This hypothesis should be tested experimentally. Synergistic interactions of ozone and oxides of sulfur have, however, been reported. Previous studies of susceptibility to airborne infection (Gardner *et al.*, 1977), tracheal glycoprotein secretion rate (Last and Cross, 1978), and pulmonary airflow resistance (Hazucha and Bates, 1975), have demonstrated that such interactions are possible, although the latter study has been disputed by more recent animal studies (Amdur *et al.*, 1978). Our quantitative morphological studies were concentrated on the pulmonary centriacinar region and hence were not designed to provide a morphological correlate of previous demonstrations of synergistic interactions of ozone and sulfuric acid based on physiological and biochemical assessments of upper airway function. Indeed morphological correlates of subtle functional alterations need not exist at the low pollutant concentrations used in this and other biologically relevant studies.

ACKNOWLEDGMENTS

The authors wish to acknowledge the technical assistance of Ms. Margaret Brummer and the advice and help given by Mr. Brian Tarkington and Dr. Daniel Chang concerning animal exposures. This work was supported by the following grants and contracts: EPA Contract 68-02-2496; NIH Grant ES00628; NIH Grant RR00169; and EPA Contract R805355010. The work partially fulfills the requirements for the degree of Doctor of Philosophy, University of California at Davis, for the senior author.

REFERENCES

ADAMSON, I. Y. R., and BOWDEN, D. H. (1977). Origin of ciliated alveolar epithelial cells in bleomycin-induced lung injury. *Amer. J. Pathol.* **87**, 569–580.

Air Resources Board: Air Quality Data. (1974). *Calif. Air Quality Data* 6(3), 16–41.

AMDUR, M. O. (1970). The impact of air pollutants on physiologic responses of the respiratory tract. *Proc. Amer. Philos. Soc.* **114**(1), 3–8.

AMDUR, M. O. (1974). 1974 Cummings memorial lecture. The long road from Donora. *Amer. Ind. Hyg. Assoc. J.* **35**, 589–597.

AMDUR, M. O., UGRO, V., and UNDERHILL, D. W. (1978). Respiratory response of guinea pigs to ozone alone and sulfur dioxide. *Amer. Ind. Hyg. Assoc. J.* **39**, 958–961.

BARROW, C. S., and DODD, D. E. (1979). Ammonia production in inhalation chambers and its relevance to chlorine inhalation studies. *Toxicol. Appl. Pharmacol.* **49**, 89–96.

BARROW, C. S., and STEINHAGEN, W. H. (1980). NH_3 concentrations in the expired air of the rat: Importance to inhalation toxicology. *Toxicol. Appl. Pharmacol.* **53**, 116–121.

BOORMAN, G. A., SCHWARTZ, L. W., and DUNGWORTH, D. L. (1980). Pulmonary effects of prolonged ozone insult in rats. Morphometric evaluation of the central acinus. *Lab. Invest.* **43**, 108–115.

BOYDEN, E. A., and TOMPSETT, T. H. (1961). The postnatal growth of the lung in the dog. *Acta Anat.* **47**, 185–215.

BOYDEN, E. A., and TOMPSETT, T. H. (1965). The changing patterns in the developing lungs of infants. *Acta Anat.* **61**, 164–192.

BRUCH, J., and SHLIPKÖTER, H.-W. (1973). Veränderungen der Lungenalveolen bei der Maus nach chronischer Exposition mit Ozon in niedriger Konzentration. *Virchows Arch. (Pathol. Anat.)* **358**, 355–368.

BURRI, P. H. (1974). The postnatal growth of the rat lung. III. Morphology. *Anat. Rec.* **180**, 77–98.

BURRI, P. H., DBALY, J., and WEIBEL, E. R. (1974). The postnatal growth of the rat lung. I. Morphometry. *Anat. Rec.* **178**, 711–730.

CAVENDER, F. L., STEINHAGEN, W. H., ULRICH, C. E., BUSEY, W. M., COCKRELL, B. Y., HASEMAN, J. K., HOGAN, M. D., and DREW, R. T. (1977). Effects in rats and guinea pigs of short-term exposures to sulfuric acid mist, ozone, and their combination. *J. Toxicol. Environ. Health* **3**, 521–533.

CAVENDER, F. L., SINGH, B., and COCKRELL, B. Y. (1978). The effects in rats and guinea pigs from 6 months exposures to sulfuric acid mist, ozone, and their combination. *J. Environ. Pathol. Toxicol.* **2**, 485–492.

CHANG, D. P. Y., and TARKINGTON, B. K. (1977). Experience with a high output sulfuric acid aerosol generator. *Amer. Ind. Hyg. Assoc. J.* **88**, 493–497.

CHANG, D. P. Y., TARKINGTON, B. K., and DUVALL, T. R. (1978). Generation of nuceli-mode sulfuric acid aerosols for health effects studies. *J. Air Pollut. Control Assoc.* **28**, 1137–1139.

DUNGWORTH, D. L., CROSS, C. E., GILLESPIE, J. R., and PLOPPER, C. G. (1975). The effects of ozone on animals. *In* "Ozone Chemistry and Technology, a Review of the Literature 1961–1974" (J. S. Murphy and J. R. Orr, eds.), pp. 29–54. Franklin, Philadelphia.

GARDNER, D. E., MILLER, F. J., ILLING, J. W., and KIRTZ, J. M. (1977). Increased infectivity with exposure to ozone and sulfuric acid. *Toxicol. Lett.* **1**(2), 59–64.

HAGEN-SMIT, A. J. (1952). Chemistry and physiology of Los Angeles smog. *Ind. Eng. Chem.* **44**, 1342–1346.

HAZUCHA, M., and BATES, D. V. (1975). Combined effect of ozone and sulphur dioxide on human pulmonary function. *Nature (London)* **257**, 50–51.

HINNERS, R. G., BURKART, J. K., and PUNTE, C. L. (1968). Animal inhalation exposure chambers. *Arch. Environ. Health* **16**, 194–206.

JUHOS, L. T., EVANS, M. J., MUSSENDEN-HARVEY, R., FURIOSI, N. H., LAPPLE, C. E., and FREEMAN, G. (1978). Limited exposure of rats to H_2SO_4 with and without O_3. *J. Environ. Sci. Health* C13(1), 33–47.

KLEINERMAN, J., and COWDREY, C. R. (1966). The effects of continuous high level nitrogen dioxide on hamsters. *Yale J. Biol. Med.* **40**, 579–585.

LARSON, T. V., COVERT, D. S., FRANK, R., and CHARLSON, R. J. (1977). Ammonia in the human airways: Neutralization of inspired acid sulfate aerosols. *Science* **197**, 161–163.

LAST, J. A., and CROSS, C. E. (1978). A new model for health effects of air pollutants: Evidence for synergistic effects of mixtures of ozone and sulfuric acid aerosols on rat lungs. *J. Lab. Clin. Med.* **91**, 328–339.

LOSCUTOFF, S. M., BURTON, F. G., and KILLAND, B. W. (1978). Neutralization of inhaled sulfuric acid aerosols by ammonia in the lung. *Fed. Proc.* **37**(3), 867.

LUM, H., SCHWARTZ, L. W., DUNGWORTH, D. L., and TYLER, W. S. (1978). A comparative study of cell renewal after exposure to ozone or oxygen. Response of terminal bronchiolar epithelium in the rat. *Amer. Rev. Resp. Dis.* **118**(2), 335–345.

NETTESHEIM, P., and SZAKAL, A. K. (1972). Morphogenesis of alveolar bronchiolization. *Lab. Invest.* **26**, 210–219.

PENHA, P. D., and WERTHAMER, S. (1974). Pulmonary lesions induced by long-term exposure to ozone. II. Ultrastructure observations of proliferative and regressive lesions. *Arch. Environ. Health* **29**, 282–289.

PIERSON, W. R., HAMMERLE, R. H., and KUMMER, J. T. (1974). "Sulfuric Acid Aerosol Emissions from Catalyst-Equipped Engines," pp. 1233–1238. SAE Paper 740287, SAE Meeting, Detroit, Mich., February 25–March 1.

SCHERLE, W. (1970). A simple method for volumetry of organs in quantitative stereology. *Mikroskopie* **26**, 57–60.

SCHWARTZ, L. W., DUNGWORTH, D. L., MUSTAFA, M. G., TARKINGTON, B. K., and TYLER, W. S. (1976). Pulmonary responses of rats to ambient levels of ozone. Effect of 7-day intermittent or continuous exposure. *Lab. Invest.* **34**, 565–578.

SCHWARTZ, L. W., MOORE, P. F., CHANG, D. P., TARKINGTON, B. K., DUNGWORTH, D. L., and TYLER, W. S. (1977). Short-term effects of sulfuric acid aerosols on the respiratory tract. A morphological study in guinea pigs, mice, rats and monkeys. *In* "Biochemical Effects of Environmental Pollutants" (S. D. Lee, ed.), pp. 257–271. Ann Arbor Science Publishers, Inc., Ann Arbor, Mich.

STEPHENS, R. J., SLOAN, M. F., EVANS, M. J., and FREEMAN, G. (1974). Early response of lung to low levels of ozone. *Amer. J. Pathol.* **74**(1), 31–58.

WERB, Z., and GORDON, S. (1975a). Secretion of a specific collagenase by stimulated macrophages. *J. Exp. Med.* **142**, 346–360.

WERB, Z., and GORDON, S. (1975b). Elastase secretion by stimulated macrophages. Characterization and regulation. *J. Exp. Med.* **142**, 361–377.

WHITE, R., LIN, H.-S., and KUHN, C., III. (1977). Elastase secretion by peritoneal exudative and alveolar macrophages. *J. Exp. Med.* **146**, 802–808.

ZITNIK, L. A., SCHWARTZ, L. W., MCQUILLEN, N. K., ZEE, Y. C., and OSEBOLD, J. W. (1978). Pulmonary changes induced by low-level ozone: Morphological observations. *J. Environ. Pathol. Toxicol.* **1**, 365–376.